IMPROVEMENT OF ENGINEERED THERMOSTABLE XYLANASE PRODUCTION VIA IMMOBILIZATION OF RECOMBINANT *ESCHERICHIA COLI* ONTO GRAPHENE OXIDE

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For my beloved husband (Nuur Muhaimin Bin Hussin), parents (Nor Ashikin Bin Abu Bakar and Siti Junaidah Bt Ithnin), my brothers (Mohd Syafiq Faiz Bin Nor Ashikin, Muhammad Syarafi Iqbal Bin Nor Ashikin and Muhammad Safiy Haziq Bin Nor Ashikin), Sister (Nurin Syahindah Syasya Binti Nor Ashikin), I dedicated this work in sincere gratitude for their patience, love and support.

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ABSTRACT

Escherichia coli is the preference host system for enzyme production by recombinant DNA technology. This was due to its promising trait that is suitable for genetically modify and the obtainability of a difference *E. coli* strains. But, the main downside when utilizing *E. coli* as a host is bacterial cell lysis due to the pressure build-up through overproduction of the expressed recombinant enzyme in the periplasmic space. Thus, the utilization of the immobilization in targeting the recombinant enzyme expression in the culture medium, presents substantial preferences over cytoplasmic excretion. Immobilization process can be applied to optimize the operational performance system of cell for industrial applications which leads to the development of economically and ecologically available enzyme such as xylanase. In this study, the effects of graphene oxide (GO) on xylanase excretion and β-galactosidase activity of immobilized *E. coli* was examined. The experiments were performed under the optimized RSM conditions (20 ºC, 0.5 mM, IPTG and pH 7) using shake flask cultivation followed by bioreactor conditions (20 ºC, 0.5 mM, IPTG and pH 7) in batch fermentation. The immobilized cells onto GO using shake flask cultivation exhibit about 3.8-fold increase in xylanase production (0.4821 U/ml) or equal to 73.7% higher compared to free cells (0.1264 U/ml). The *E. coli* were also demonstrated 15.3% reduction of cell lysis (0.0299 U/ml of β-galactosidase activity) compared to free cells (0.1273 U/ml of β-galactosidase activity) and 72.6% increase in plasmid stability compared to free cells. The xylanase concentration was 0.482 U/ml, representing 99.4% of the predicted value (0.485 U/ml) and 1.71-fold higher than the value before optimization process (0.283 U/ml). The occurrence of cell lysis demonstrated 99.3% reduction and increased in the plasmid stability up to 87% under the optimized RSM condition compared to free cells. The stirrer tank bioreactor also showed 5% higher in xylanase excretion (0.506 U/ml) with 33% reduction of β-galactosidase activity (0.06 U/ml) compared to shake flask cultivation (0.09 U/ml) after 24h. While from the Liquid Chromatography Mass Spectometry (LCMS) analysis showed that the xylooligosaccharides was found to be hydrolyzed by an endoxylanase to produce xylopentaose sugar. Hence, this study demonstrated that the immobilization of *E. coli* on GO potentially to be valuable for the excretion of recombinant proteins in *E. coli* with high cell viability.

ABSTRAK

Escherichia coli adalah sistem hos utama untuk penghasilan enzim menggunakan teknologi rekombinan DNA disebabkan ciri-ciri yang memberangsangkan untuk pengubahsuaian genetic juga mudah untuk didapati. Namun, kelemahan utama apabila menggunakan *E. coli* sebagai hos adalah pemecahan sel bakteria; akibat tekanan yang terbina melalui pengeluaran enzim secara berlebihan dalam ruang periplasma. Penggunaan pendekatan imobilisasi dalam mensasarkan penghasilan enzim rekombinan dalam medium kultur, menjadi pilihan utama berbanding perkumuhan sitoplasma. Proses imobilisasi boleh digunakan untuk sel bagi mengoptimumkan sistem operasi untuk aplikasi industri yang membawa kepada pembangunan biomangkin ekonomi dan ekologi enzim seperti xylanase. Dalam projek ini, kesan oksida grafit (GO) pada rembesan xylanase dan aktiviti β-galactosidase dari *E. coli* telah diamati. Kajian ini telah dilakukan di bawah optimum kondisi RSM (20 °C, 0.5 mM IPTG dan pH 7) menggunakan kaedah kelalang bergoncang diikuti oleh fermentasi dalam bioreactor (20 °C, 0.5 mM IPTG dan pH 7) dengan menggunakan kultur *batch.* Sel immobilisasi menggunakan nanopartikel GO mempamerkan peningkatan 3.8 kali ganda dalam pengeluaran xylanase (0.4821 U/ml) atau bersamaan dengan 73.7% lebih tinggi berbanding sel bebas (0.1264 U/ml). Immobilisasi sel juga menunjukkan pengurangan kadar sel pecah sebanyak 15.3% (0.0299 U/ml daripada aktiviti β-galactosidase) berbanding sel bebas (0.1273 U / ml aktiviti β-galactosidase) dan peningkatan dalam kestabilan plasmid sebanyak 72.6% berbanding sel bebas. Xylanase mempamerkan kepekatan sebanyak 0.482 U/ml, mewakili 99.4% daripada nilai yang diramalkan (0.485 U/ml) dan 1.71 kali ganda lebih tinggi daripada nilai sebelum proses pengoptimuman (0.283 U/ml). Kadar lisis sel menunjukkan pengurangan 99.3% dan peningkatan dalam kestabilan plasmid sehingga 87% di bawah kondisi optimum RSM berbanding sel bebas. Agitasi bioreaktor juga menunjukkan peningkatan perembesan xylanase sebanyak 5% lebih tinggi dalam pengeluaran xylanase (0.506 U/ml) dengan pengurangan aktiviti β-galactosidase aktiviti sebanyak 33% (0.06 U/ml) berbanding kelalang kon; (0.09 U/ml) selepas 24 jam. Manakala melalui Kromatografi Cair-Spektrometri Massa (LCMS) menunjukkan bahawa xylooligosakarida didapati dihidrolisis oleh endoxylanase untuk menghasilkan gula xylopentaosa. Jadi, kajian ini membuktikan immobilisasi *E. coli* pada GO berguna untuk perembesan rekombinan protin dalam *E. coli* dengan kestabilan sel yang tinggi.

ABSTRACT

Escherichia coli is the preference host system for enzyme production by recombinant DNA technology. This was due to its promising trait that is suitable for genetically modify and the obtainability of a difference *E. coli* strains. But, the main downside when utilizing *E. coli* as a host is bacterial cell lysis due to the pressure build-up through overproduction of the expressed recombinant enzyme in the periplasmic space. Thus, the utilization of the immobilization in targeting the recombinant enzyme expression in the culture medium, presents substantial preferences over cytoplasmic excretion. Immobilization process can be applied to optimize the operational performance system of cell for industrial applications which leads to the development of economically and ecologically available enzyme such as xylanase. In this study, the effects of graphene oxide (GO) on xylanase excretion and β-galactosidase activity of immobilized *E. coli* was examined. The experiments were performed under the optimized RSM conditions (20 ºC, 0.5 mM, IPTG and pH 7) using shake flask cultivation followed by bioreactor conditions (20 ºC, 0.5 mM, IPTG and pH 7) in batch fermentation. The immobilized cells onto GO using shake flask cultivation exhibit about 3.8-fold increase in xylanase production (0.4821 U/ml) or equal to 73.7% higher compared to free cells (0.1264 U/ml). The *E. coli* were also demonstrated 15.3% reduction of cell lysis (0.0299 U/ml of β-galactosidase activity) compared to free cells (0.1273 U/ml of β-galactosidase activity) and 72.6% increase in plasmid stability compared to free cells. The xylanase concentration was 0.482 U/ml, representing 99.4% of the predicted value (0.485 U/ml) and 1.71-fold higher than the value before optimization process (0.283 U/ml). The occurrence of cell lysis demonstrated 99.3% reduction and increased in the plasmid stability up to 87% under the optimized RSM condition compared to free cells. The stirrer tank bioreactor also showed 5% higher in xylanase excretion (0.506 U/ml) with 33% reduction of β-galactosidase activity (0.06 U/ml) compared to shake flask cultivation (0.09 U/ml) after 24h. While from the Liquid Chromatography Mass Spectometry (LCMS) analysis showed that the xylooligosaccharides was found to be hydrolyzed by an endoxylanase to produce xylopentaose sugar. Hence, this study demonstrated that the immobilization of *E. coli* on GO potentially to be valuable for the excretion of recombinant proteins in *E. coli* with high cell viability.

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LIST OF SYMBOLS AND ABBREVIATIONS

g	-	Gram
h	-	Hour
l	-	Liter
min	-	Minute
ml	-	Milliliter
sec	-	Second
rpm	-	Revolution per minutes
E. coli	-	*Escherichia coli*
Sp.	-	Species
CCD	-	Central Composite Design
DO	-	Dissolved Oxygen
FFD	-	Full Factorial Design
CNT	-	Carbon Nanotube
LB	-	Luria Bertani
GO	-	Graphene Oxide
ONPG	-	*Ortho*-nitrophenyl-β-galactoside
RSM	-	Response Surface Methodology
ANOVA	-	Analysis of Variance
DNA	-	Deoxyribonucleic Acid
OFAT	-	One Factor at One Time
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
LCMS	-	Liquid Chromatography Mass Spectrometry
FESEM	-	Field Emission Scanning Electron Microscopy

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Recombinant DNA technology has facilitated researchers to bring an extensive amount of various proteins and enzymes in microbes that were hard to gain in quantity or relatively high-priced. The foreign genes expression has been recorded in a range of microorganisms such as fungi and bacteria by cloning the desired gene into the plasmid vector under the regulation of an inducible promoter such as isopropyl β-D-1-thiogalactopyranoside (Kuniechick *et al*., 2009). The expression of recombinant enzyme is recognized as heterologous protein; and the most frequently utilized expression systems for heterologous gene production of both prokaryotic and eukaryotic origins is microbial expression system (Fakruddin *et al*., 2013).

The usage of plasmid as a vector for production of recombinant enzyme or protein is vital to produce high beneficial enzymes, in adequate supply for research and industrial prospect (Joseph *et al*., 2015). However, in commercial production of recombinant microorganisms, the plasmid instability has become one of the major issues during this process. The plasmid instability is an inclination of the transformed cells to lose their engineered characteristics by alteration or loss of plasmid. As a consequence, it may impact in a critical loss in targeting recombinant protein throughput and the most often utilized bacterial expression system to produce the heterologous protein is *E. coli* (Fakruddin *et al*., 2013; Kuniechick *et al*., 2009).

Escherichia coli is Generally Recognized as Safe (GRAS) organism and verified to be a cost-effective host cell line for proteins and enzymes production by recombinant DNA technology, due to its promising traits such as fast and high cell growth, easy to cultivate, low cost, suitable and simple for genetically modify and the obtainability of a different *E. coli* strains (Rosano & Ceccarelli, 2014). However, in the process, tons of

issues can possibly happen, such as inactivation of the protein enzyme, formation of the inclusion body (IB) and unfavorable cell growth rate are certain complications that frequently occurred (Rosano & Ceccarelli, 2014; Birkholtz *et al*., 2008).

Throughout the expression of heterologous protein, the composition of insoluble aggregates or lower production might result from the dissimilarities in identical codon usage of expression host. Other than that, the protein overexpression in *E. coli* could also lead to insoluble protein synthesis in the form of inclusion bodies, caused by metabolic burden. When a foreign DNA is brought into the bacterial strain, the new synthesis recombinant polypeptide will be expressed in the microenvironments of *E. coli*. This expression might vary from the initial condition relating to osmolarity, cofactors, folding mechanisms, pH, redox potential and both spatial and temporally control of gene expression is disoriented (Rosano & Ceccarelli, 2014). Hence, the desired protein production involves bioprocess media optimization for targeting the high cell density culture. By determining the optimum cultivation condition such as medium type for microorganism can reduce the metabolic stress exposed to the host cells (Angov *et al*., 2008; Birkholtz *et al*., 2008).

Thus, it is worthy to discover the optimum medium for cultivation system such as by providing the enriched culture medium for higher soluble proteins expression. There are several types of media have been used for cultivation such as, Terrific Broth media, salt media and Luria-Bertani broth (Baser *et al*., 2010). While, the Luria-Bertani broth is the most regularly utilized medium culture for *E. coli* as it contains high nutrients, measures simple preparation and optimum osmolarity for the early exponential growth phase. All these aspects make it sufficient for enzyme production and recompense for the statement claiming it is not the best preference for attaining high cell density cultures (Sezonov & Ari, 2007).

On another point of view, to improve the immobilization process for better operational performance system of recombinant *E. coli* and with the development of nanotechnology, it is now possible for nanoparticles to be used as immobilization matrix for a wide range of industrial applications such as production of recombinant protein by immobilized bacteria. This is due to nanoparticle's specific physico-chemical properties such as its nano scale size, chemical composition and surface area that make it suitable material to fix diffusion problems when the process is dealing with macromolecular substrates. Moreover, a large surface-to-volume proportion presented by nanomaterial resulted in the higher concentration of the immobilized cells compared to those gave by other materials (Ahmad & Sardar, 2015; Saifuddin *et al*., 2016). Thus, nanoparticles such

as graphene oxide and carbon nanotube are key modules in the future market of advance innovation that greatly impact the material's mechanical properties as well as biocompatible environment or surrounding area for immobilization method (Ahmad & Sardar, 2015).

Concurrently, the substrate utilized for this research; polysaccharides of xylan are generally correlated with lignin as well as cellulose, which act as an essential structural support to build up the plant cell walls. The degradation of hemicellulose constituent, predominantly comprised of xylan, necessitates effective xylanolytic enzymatic systems involving certain enzymes such as β-xylosidase, endo-β-1,4-xylanase, α-glucuronidase, α-L-arabinofuranosidase, p-coumaric acid esterase, acetyl xylan esterase and ferulic acid esterase. Recently, there has been a rising attention in xylanase enzyme fermentations concerning their wide-ranging applications in a number of industries, particularly in the pulp and paper commerce (Rusli *et al*., 2009; Basar *et al*., 2010).

Therefore, xylanases enzymes accountable for the hydrolysis of xylan to xylooligosaccharides (XOS) and have drawn intentness by virtue of their prospective in numerous industrial progresses. Moreover, xylose is the primary constituent of the hemicellulose xylan and the secondary most sufficient plant biomass derivative sugar (Salamanca *et al*., 2014). Xylanase in combination with other enzymes, act efficiently to degenerate xylan to sugar constituents which can be produce naturally by wild type filamentous fungi species such as *Trichoderma sp.* and *Aspergillus sp.* Xylanase can be applied in wide-ranging utilization for industrial scales such as to enhance the quality of bread, improve the digestibility of ruminant feeds and as a pre-bleaching of kraft pulp (Farliahati *et al*., 2010).

In this study, the graphene oxide was chosen as a matrix for recombinant *E. coli* to improve the engineered thermostable xylanase excretion with fewer occurrences of cell lysis. Furthermore, a thorough search for relevant literatures did not yield any related article and to the extent of our knowledge, there have been no studies on excretion of engineered thermostable xylanase by recombinant *E. coli* that have been immobilized through an adsorption of the cells using graphene oxide.

1.2 Problem statement

The establishment of heterologous protein by employing recombinant DNA innovation has drawn a great interest in the industrial process of biotechnology and the manufacturing level (Man *et al*., 2015; Mergulhão *et al*., 2005). However, despite

impressive progress in employing the *E. coli* cell factory for excretory production of recombinant enzymes, the production of expressed protein has encountered with the certain operative obstacle during the process (Yoon *et al*., 2010).

Firstly, the complications in recovering excretion of correctly folded proteins; as the overexpression of proteins usually occur in the inclusion bodies (IB) form from the active proteins that could only be recovered by a complex and expensive process (Choi & Lee, 2004). Secondly, the excretion yield of recombinant protein was usually untraceable or low-set; mainly because of interactions within the hydrophobic surface regions of protein causing aggregates (Yoon *et al*., 2010). Thirdly, the autolysis of cell due to overexpression of recombinant protein in host cell (Choi & Lee, 2004).

Accordingly, assortment techniques have been utilized and established to resolve these issues; for instance, lowering the concentration of inducer by controlling expression level using various promoters, excretion of recombinant proteins into the culture medium or periplasmic space, use of various host strains and certain recombinant proteins to induce modulation of chaperones and a class of proteins that assist protein folding (Wyre & Overton, 2014; Choi & Lee, 2004). The other commonly suitable method to maximize recombinant protein solubility and folding is by reducing the imposed stress on bacteria and lowering the rates of recombinant protein excretion by controlling the growth temperature. This is to enable the translation and folding process occurs more gradually in recombinant protein (Wyre & Overton, 2014).

Moreover, the declined yields of a target protein through the gene expression of *E. coli* are regularly caused by the metabolic stress and consequence in the reduced cell growth and redirection of cellular metabolic process. In certain cases, it has been described to occur caused by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) inducer. Specifically, once induction is initiated when a certain critical cell mass is obtained. This is probably caused by the deficiency of essential metabolites during process (Ramchuran *et al*., 2005). Furthermore, the cultural conditions and environmental factors such as temperature have the main influence on the total protein expression and cell metabolism (Morowvat *et al*., 2015).

Thus, in order to improve the system performance of the recombinant *E. coli* for recombinant protein excretion, immobilization of cell was used. Cell immobilization could reduce the plasmid instability and cell lysis issues, improving the productiveness of the recombinant cells, low sensitivity to pH and temperature when compared with free cells. Thus, will lowering the possibility of contamination, decreasing the fermentation time, advancing the rate of substrate uptake, and resultant in estimated concentration of

volatile component for product. Meanwhile, studies on finding the convenient method to improve the xylanase enzyme production that being recognized as high potential enzyme for wide range application of industrial use such as pulp and paper industry is still deliberately studied by researchers. Consequently, this study demonstrates that graphene oxide is a convenient immobilization matrix for recombinant *E. coli* due to its unique features as well as special characteristics to enhance the immobilization cell efficiency and excrete high xylanase expression.

1.3 Objectives of the study

1. To improve the engineered thermostable xylanase excretion of immobilized recombinant *E. coli* onto graphene oxide in comparison with free cell.
2. To determine the cell viability with high plasmid stability of immobilized recombinant *E. coli* onto graphene oxide.

1.4 Scopes of the study

1. Screening the effect of the cultural conditions (nanomatrix quantity, expression medium, IPTG, post induction temperature, post induction time, agitation rate, inoculum size and pH) on improvement of xylanase excretion and cell viability of the immobilized cell using one factor at one-time method (OFAT).
2. Screening the effect of significant factors of cultural conditions (IPTG, post induction temperature, post induction time, agitation rate, inoculum size and pH) on the immobilized cell for improvement of xylanase excretion and cell viability using Design-Expert Software; full factorial design (FFD).
3. Optimization of the cultural conditions (IPTG, post induction temperature and post induction time) on the excretion of recombinant xylanase and reduction of cell lysis using Design-Expert Software; Response Surface Methodology (RSM).
4. Production of high level xylanase excretion of immobilized cell in the 5 L bioreactor by using optimum RSM conditions.
5. Determination of immobilized cell's enzymatic production of xylooligosaccharides (XOS) from xylan using Liquid Chromatography-Mass Spectrometry (LCMS).

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